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Blockade of the cholinergic system during sensitization enhances lung responsiveness to allergen in rats

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Summary

Although acute prophylactic administration of atropine modulates airway responsiveness, the role of the parasympathetic nervous system in the pathogenesis of sensitization and in antigen-induced bronchoconstriction remains unclear. The aim of the present study is to determine whether blocking muscarinic receptors during chronic allergen exposure modulates lung responsiveness to the specific allergen. Forty rats were randomly assigned to one of the following five treatment groups: sensitization with saline vehicle, intraperitoneal injection of ovalbumin (1 mg) with or without atropine treatment (10 mg/kg per day) and repeated ovalbumin aerosol (1.25 mg/mL for 20 minutes) either alone or combined with atropine. Lung responsiveness to methacholine (4–16 μg/kg per minute) and intravenous ovalbumin (2 mg) was established before and 21 days after treatment with forced oscillations following bilateral vagotomy. Lung cellularity was determined by analysis of bronchoalveolar lavage fluid (BALF). A lung inflammatory response in all sensitized animals was defined as an increase in the number of inflammatory cells in the BALF. Baseline respiratory mechanics and methacholine responsiveness on Days 0 and 21 were comparable in all groups. However, increases in airway resistance following intravenous allergen challenge were significantly exacerbated in rats that received atropine. Inhibition of the cholinergic nervous system during allergic sensitization potentiates bronchoconstriction following exposure to the specific allergen. These findings highlight the role of the cholinergic neuronal pathway in airway sensitization to a specific allergen.

KEYWORDS

airway hyperresponsiveness, allergen exposure, animal models, asthma

1 | INTRODUCTION

Worldwide, increasing exposure to allergens and air pollution is contributing to a marked increase in the incidence of chronic respiratory diseases, which are becoming a major public health challenge.[1,2] One of the main adverse features of these chronic diseases is bronchial hyperresponsiveness (BHR) to different exogenous constrictor stimuli, which precipitates exacerbation of lung function. Although many studies have attempted to clarify the pathophysiological mechanisms primarily involved in the occurrence of BHR,[3] the specific pathways leading to chronic airway inflammation have not been fully elucidated.

New concepts have recently emerged that explain the importance of the autonomic nervous system in generating neurogenic

inflammation as a major contributor to airflow limitation. In the concept of neurogenic inflammation, neural mediators are considered to contribute to the inflammatory microenvironment responsible for the airflow limitation that causes chronic pulmonary symptoms.[4-7] Vagal afferent nerves in the lung play a significant role in modulating airway tone, with the bronchopulmonary sensory C-fibres being of particular importance.[8-11] These C-fibres are very sensitive to various endogenous inflammatory mediators and inhaled irritants.[8-10] Both vagal control and afferent sensory C-fibres have been demonstrated to play key roles in antigen-induced bronchoconstriction in sensitized animals[12-14]; however, the involvement of the parasympathetic pathway during the sensitization period has not been characterized.

Thus, the aim of the present study was to characterize the general involvement of the cholinergic neural pathway in the development of BHR during allergen exposure. An earlier report suggesting that acute inhibition of the parasympathetic pathway before allergen challenge increased pulmonary inflation pressure[15] led us to hypothesize that blockade of the parasympathetic pathway by blocking muscarinic receptors during allergen sensitization may modulate lung responsiveness to the specific allergen. In addition, to reveal the relative contributions of the central and peripheral lung to adverse allergen-induced changes in lung function, we assessed airway and tissue responses separately by using the forced oscillation technique (FOT).

2 | RESULTS

The present study was performed on 40 rats divided into five groups: a vehicle control group (C, n = 8), two groups receiving intraperitoneal injections of ovalbumin (OVA) with (S, n = 7) or without atropine (O, n = 9) and a final two groups receiving repeated OVA aerosol either alone (OA, n = 7) or combined with atropine (SA, n = 9).

2.1 | End-expiratory lung volume

The results of end-expiratory lung volume (EELV) measurements normalized against bodyweight are shown in Figure 1. There were no differences in EELV between Days 0 and 21 within Groups C, O and S as evaluated by two-way repeated measures analysis of variance (ANOVA). However, in groups receiving atropine (Groups OA and SA), there was a significant increase in EELV on Day 21 compared with Day 0 ($P = 0.02$ and $P = 0.01$, respectively).

2.2 | Methacholine challenge

Respiratory mechanical changes in response to methacholine (MCh) challenge on Days 0 and 21 in each group are shown in Figure 2. According to two-way repeated measures ANOVA, there was a significant interaction between group allocation and MCh responses in terms of airway resistance (Raw) and respiratory tissue damping (G) on Day 21 ($P < 0.002$ and $P < 0.001$, respectively), indicating that these treatments affected lung responsiveness to MCh. Although MCh caused marked dose-dependent (4–16 μg/kg per minute) increases in Raw and G, the MCh-induced increases in respiratory tissue elastance (H) were mild but significant ($P < 0.05$). On Day 0, there were no significant differences between groups in Raw, G and H responses to all MCh concentrations tested. On

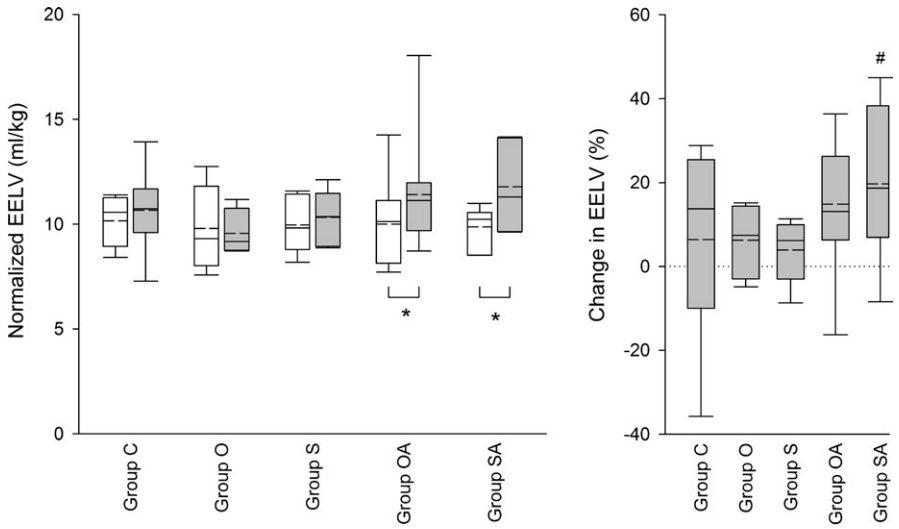

FIGURE 1 End-expiratory lung volume normalized to bodyweight in the protocol groups measured before (□, Day 0) and after (■, Day 21) the sensitization protocol with or without atropine treatment (left) and the relative changes between the two experimental days (right). Group C, control group with no treatment; Group O, a single intraperitoneal dose of ovalbumin sensitization; Group S, intraperitoneal ovalbumin with subsequent ovalbumin aerosol sensitization; Group OA, a single intraperitoneal dose of ovalbumin and continuous atropine during the sensitization period; Groups SA, intraperitoneal ovalbumin with subsequent ovalbumin aerosol sensitization and continuous atropine during the sensitization period. Horizontal continuous lines within the box mark the median values; the lower and upper boundaries of boxes indicate the 25th and 75th percentiles, respectively; the whiskers above and below the box indicate the 90th and 10th percentiles, respectively; dashed lines: mean values. *$P < 0.05$ between Days 0 and 21; #$P < 0.05$ vs zero

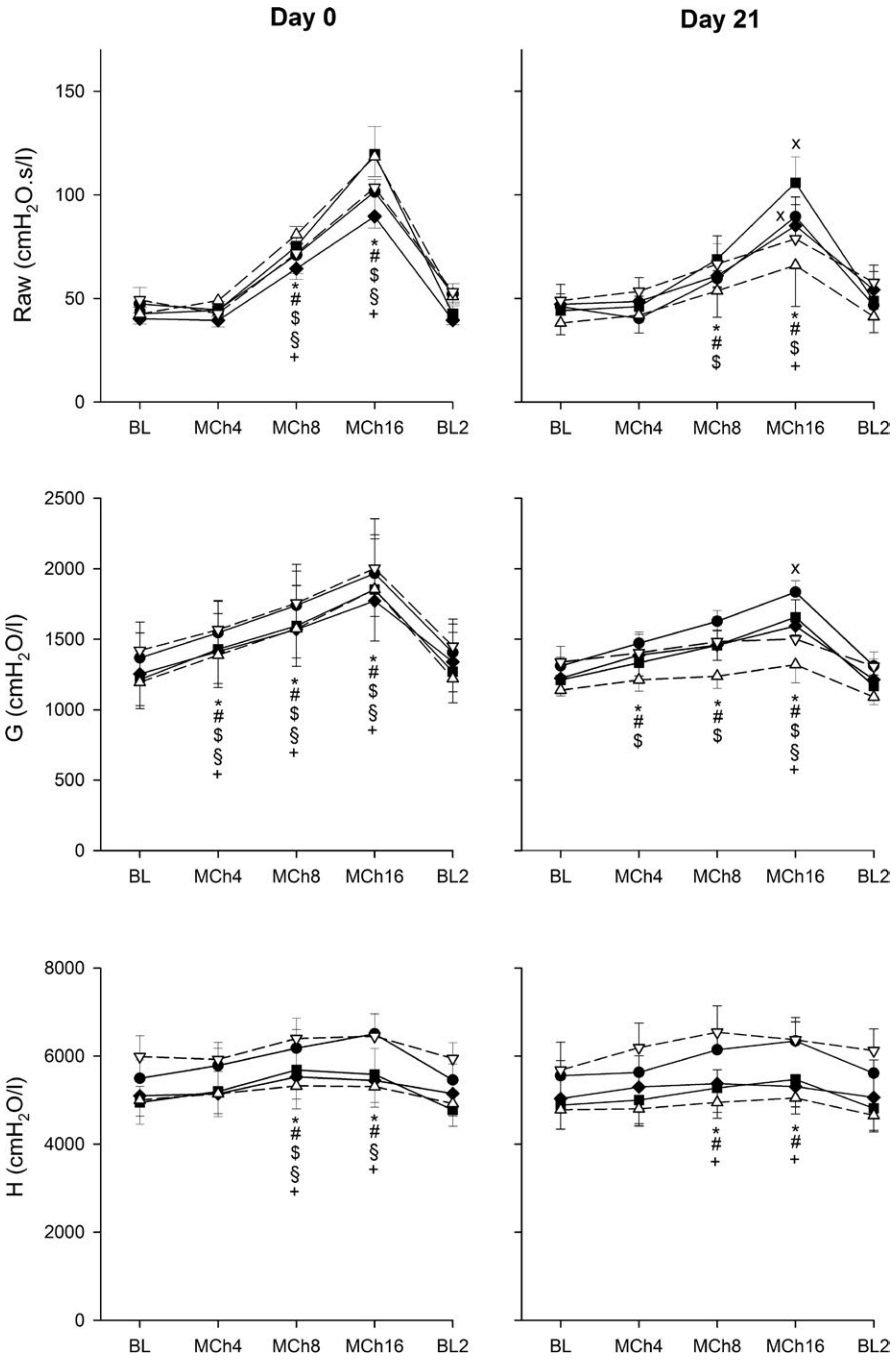

FIGURE 2 Changes in airway resistance (Raw) and in tissue damping (G) and elastance (H) in response to increasing iv doses of methacholine (MCh4-MCh16) assessed before (Day 0) and after sensitization (Day 21). *, #, $, § and + denote statistically significant differences relative to the baseline (BL) in Groups (–●–) C, (–■–) O, (–◆–) S, (-△-) OA and (-▽-)SA, respectively. Symbol x denotes statistically significant difference relative to Group OA within a condition

Day 21, rats that did not receive atropine treatment (Groups C, O and S) had similar Raw, G and H responses to MCh as seen on Day 0. However, lung responsiveness to MCh was inhibited in rats that were treated continuously with atropine (Groups OA and SA; $P < 0.05$ for all). On Day 21, there was a tendency for an increased Raw response to MCh in Group OA compared with Day 0, but the difference did not reach statistical significance ($P = 0.06$). In contrast, in Group SA, the Raw response to MCh was increased on Day 21 only for the highest concentration tested (16 μg/kg per minute; $P = 0.02$). Similar results were seen for G on Day 21 in atropine-treated rats, with the increased response to MCh only reaching statistical significance for the highest concentration tested (16 μg/kg per minute). The MCh-induced changes in H were abolished in atropine-treated rats.

2.3 | Ovalbumin challenge

Temporal changes in respiratory mechanical parameters in response to OVA are shown in Figure 3. Two-way repeated measures ANOVA revealed significant interactions between group allocation and OVA-induced Raw, G and H responses ($P < 0.001$, $P < 0.001$ and $P < 0.05$, respectively), indicating that the treatments affected lung responsiveness to OVA. According to post hoc analyses, there were no significant changes in any parameters

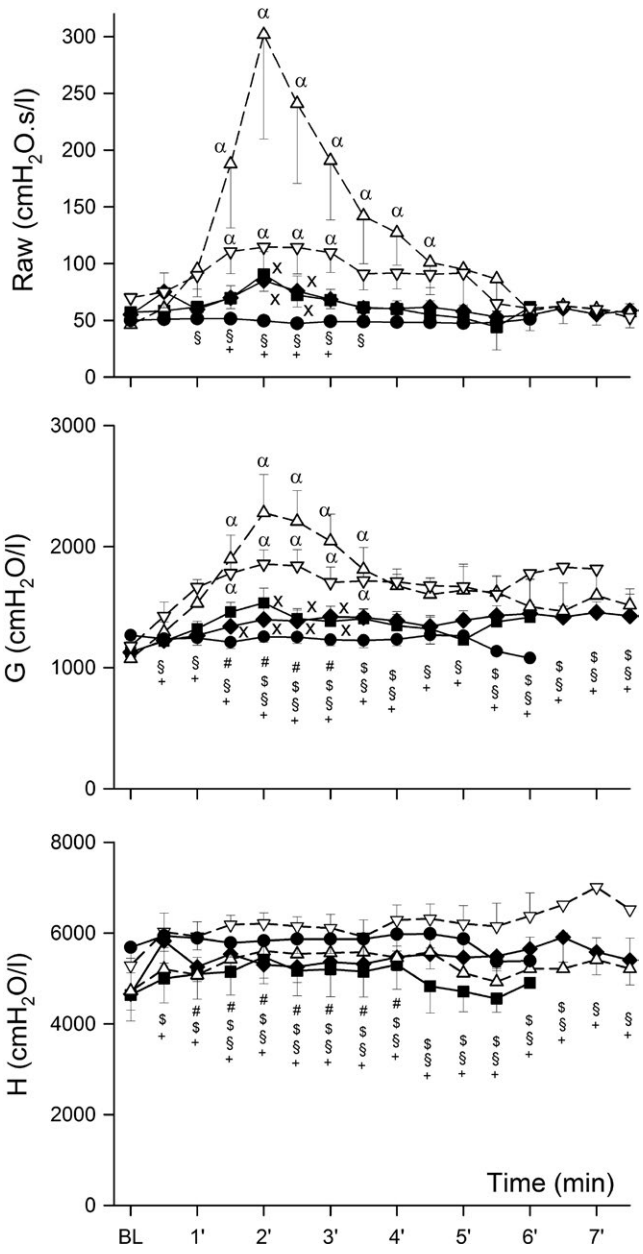

FIGURE 3 Changes in airway resistance (Raw) and in tissue damping (G) and elastance (H) in response to the specific allergen (ovalbumin). *, #, $, § and + denote statistically significant differences relative to the baseline (BL) in Groups (–●–) C, (–■–) O, (–◆–) S, (-△-) OA and (-▽-) SA, respectively. α and x denote statistically significant difference relative to Groups C and OA, respectively, within a condition

in response to OVA in Group C. Peak increases in the parameters evaluated following OVA challenge were seen approximately 2–3 minutes after OVA injection in the sensitized rats. Moderate responses were observed in Group O and S rats, with mean (±SEM) peak percentage Raw responses in these groups of 62 ± 18% and 63 ± 34%, respectively, compared with 11.9 ± 4.2% in Group C; these differences did not differ significantly because of considerable interindividual variability ($P = 0.12$). However, marked and sustained OVA-induced increases in Raw were observed in both atropine-treated groups, with peak Raw changes of 657 ± 245% and 177 ± 47% in Groups OA and SA, respectively ($P < 0.05$ vs Group C). Significant increases were seen in G and H 2, 3 and 4 minutes after OVA challenge in all OVA-treated groups ($P < 0.05$ for all), whereas corresponding increases in G and H were detected earlier and lasted longer in Groups S, OA and SA.

2.4 | Bronchoalveolar lavage

After the 3-week treatment period, the total number of cells in the bronchoalveolar lavage fluid (BALF) differed between groups: sensitization with OVA aerosols (Groups S and SA) resulted in increased accumulation of inflammatory cells in the bronchoalveolar space (Figure 4a; $P < 0.001$, one-way ANOVA test on ranks). Regarding differential cell counts in the BALF, significant neutrophil accumulation was observed at the expense of macrophages in OVA aerosol-treated rats (Groups S and SA; $P < 0.05$; Figure 4b). There was a tendency for an increase in the relative number of eosinophils in Groups S, OA and SA as compared to Group C, but the difference did not reach statistical significance ($P = 0.078$).

3 | DISCUSSION

The present study investigates the involvement of the cholinergic pathway during allergic airway sensitization. The results demonstrate that inhibition of the parasympathetic nervous system during the sensitization period augments lung responsiveness to the specific allergen. These enhanced responses were manifested more markedly in mechanical changes, reflecting constriction of the main conducting airways, and, to a lesser extent, in mechanical indices characterizing respiratory tissue mechanics. This exacerbation was striking in rats systemically exposed to the allergen, whereas repeated inhalation of the allergen after systemic sensitization blunted the intensified mechanical responses to the allergen. The excessive responses in respiratory mechanical parameters in rats with parasympathetic nerve blockade were associated with significant increases in the normalized static lung volume.

3.1 | Baseline measurements

There were no differences in basal airway or respiratory tissue mechanical parameters between the different groups. These findings are in agreement with previous reports of the lack of effect of OVA sensitization and atropine on baseline respiratory mechanics.[16-18] However, in the present study there was an increase in static lung volume in atropine-treated rats. This may be a consequence of the direct effect of atropine on EELV, which is in line with a previous report indicating that loss of parasympathetic control in humans leads to increased functional residual capacity.[19]

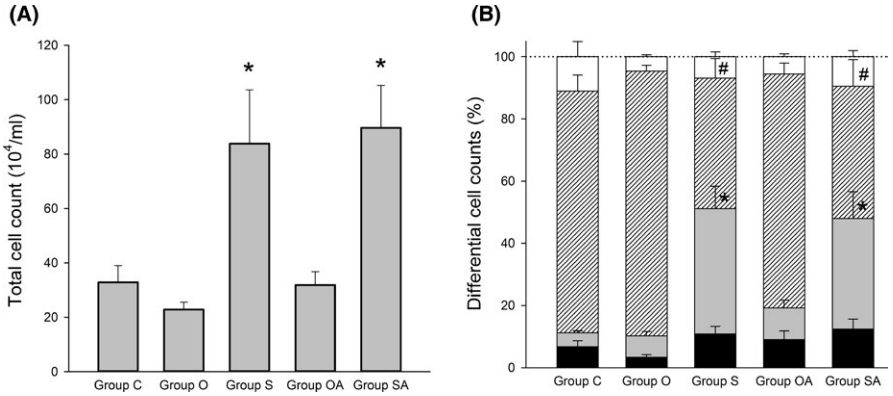

FIGURE 4 (A) Total and (B) differential cell counts in the bronchoalveolar lavage fluid in the protocol groups on Day 21. * and # denote statistically significant differences in the neutrophil and macrophage cell counts relative to those in Group C, respectively. (□) Lymphocytes, (▨) macrophages, (▩) neutrophils, (■) eosinophils

3.2 | Responses to a non-specific cholinergic agonist

The aim of the constrictor challenge with MCh was to evaluate how sensitization with OVA and prolonged atropine pretreatment affect the lung response to a non-specific cholinergic agonist. In the present study, repeated OVA aerosol exposure generated chronic airway inflammation, manifested as the accumulation of neutrophils and a tendency for an increase in the number of eosinophils. These results are in agreement with previous studies in which similar sensitization protocols were used.[15,20] However, despite these cellular changes, there was no evidence of altered lung responsiveness to MCh. Following sensitization with aerosolized OVA, the coexistence of bronchial inflammation and the lack of BHR to non-specific constrictor challenge are controversial, but our findings are in accordance with results indicating dissociated inflammatory and mechanical responses.[15,21,22]

Surprisingly, prolonged atropine treatment throughout the experimental period blunted, but did not abolish, the respiratory mechanical changes resulting from intravenous infusion of MCh. Detailed analyses of the responses in individual rats revealed that only two rats in Groups OA and SA responded to MCh in a similar way to rats that did not receive atropine, whereas all other rats (n = 5 each in Groups OA and SA) did not respond to MCh at all. These observations suggest that although the subcutaneously implanted pumps provided sufficient atropine to most rats to maintain general muscarinic receptor blockade, the pumps may have suffered a technical failure by the time of the second set of experiments in two rats from each of these group. However, the lack of weight gain in these outlier rats (data not shown) suggests that continuous atropine administration led to a hypermetabolic state in these two rats. Nevertheless, the concentration of atropine in the circulation may not have been sufficient to block responses to MCh by Day 21, when the second set of experiments was performed. Therefore, we included these animals in data analyses, bearing in mind this potential drawback for the other findings.

3.3 | Responses to the specific allergen

In agreement with previous results,[20,23] intravenous injection of the specific allergen in sensitized rats led to the development of bronchoconstriction via various mediators involved in immune and inflammatory responses. The lack of a difference in mechanical responses between Groups S and O may be attributed to the greater importance of topical administration of the allergen during the sensitization period rather than its systemic delivery. This finding is in accordance with previous results demonstrating no differences in immune responses following local and systemic administration,[24] and revealing no need for a systemic adjuvant to induce airway inflammation.[25]

The main finding of the present study is the significant exacerbation of the anaphylactic lung constrictor response in rats treated with atropine throughout the sensitization period. This effect was somewhat diminished, but still significant, in rats that were exposed repeatedly to aerosolized OVA. This blunted response may be attributed to the development of allergen tolerance in this group of rats as a consequence of a decrease in OVA-specific IgE antibodies.[26,27] Moreover, blocking vagal control of the lungs before allergen challenge ensured that the magnitude of the response was not affected by the autonomic nervous system, but rather by local and systemic mediators.

The mechanisms potentially underlying these findings can be inferred from the results of previous studies. Atropine has similar affinities for muscarinic M_2 and M_3 receptors. The M_3 receptors participate in constrictor responses of the airway smooth muscle, whereas prejunctional M_2 receptors are part of a negative feedback loop by which the contractile response is inhibited.[6] It is possible that the exaggeration of the allergen response is due to dominance of a diminished negative feedback loop following blockade of M_2 receptors rather than blockade of excitatory M_3 receptors,[15] resulting in an imbalance in the constrictor-relaxation processes. Further, because muscarinic receptors are also expressed on eosinophils[28] and because there was a tendency for an increase in the number of eosinophils in the sensitized groups in the present study, blocking the receptors involved in the negative feedback loop may have further contributed to the increased activation. In addition, the enhanced allergen response could be attributed to amplification of the interaction between the airway nerves and eosinophils mediated by nerve growth factor.[29] This concept is in line with previous results of an inhibited lung constrictor response following the selective blockade of M_3 receptors.[30,31] Together, these observations clearly

demonstrate the key role of the parasympathetic neural pathway in allergen sensitization.

3.4 | Limitations

A limitation inherent in the present study is related to differences in airway innervation between different species.[32] This variability affects the density and distribution of pre- and post-ganglionic nerve fibres, as well as the expression of nicotinic and muscarinic receptor subtypes. In addition to these anatomical differences, functional dissimilarities can also be anticipated because rat bronchi synthesize and store substantially more of the main cholinergic neurotransmitter acetylcholine than human airways.[32] These factors warrant consideration before extrapolating the findings of the present study to human lung diseases.

4 | CONCLUSIONS

The present study characterizes the potential role of the cholinergic parasympathetic nervous system in the modulation of lung responses to a specific allergen in an experimental model of airway sensitization. Although the potential benefit of anticholinergic therapy in already established asthma is not a matter of debate, concomitant blockade of muscarinic receptors during the period of sensitization resulted in enhanced lung responses to the specific allergen. This observation highlights the importance of the cholinergic pathway in the sensitization process and the involvement of vagal control in allergen-induced bronchospasm.

5 | METHODS

5.1 | Sensitization and protocol groups

The experimental protocol was approved by the Experimental Ethics Committee of the University of Geneva and the Animal Welfare Committee of the Canton of Geneva, Switzerland. Brown Norway rats (Janvier SAS, Le Genest-Saint-Isle, France), 13 weeks old, were used in the experiments. The animals were housed in a chamber with artificial light for 12 hours/day. Food and water were available ad libitum.

The rats were randomly assigned to one of five protocol groups (Figure 5). Sensitizations and treatments were started with an intraperitoneal injection of either 1 mL of normal saline in the naive rats (Group C), or ovalbumin (OVA, 1 mg; Sigma-Aldrich, Buchs, Switzerland). OVA was injected with aluminium hydroxide (50 mg; Sigma-Aldrich) as an adjuvant in the rats receiving a single dose of OVA with (Group OA) and without (Group O) atropine treatments, as well as in rats sensitized with repeated aerosols with (Group SA) or without atropine (Group S). Twenty-minute aerosol exposures were daily applied for 5 days between Days 14 and 20 either with normal saline in the control group of rats (Group C) and in Groups O and OA, or with OVA (25 mg in 20 mL normal saline) in the sensitized animals in Groups S and SA.

Animals were placed two at a time in an exposure chamber connected to a nebulizer (Syst'AM, Villeneuve-sur-Lot, France; type LS 2000-3011). In the animals in the two protocol groups in which the parasympathetic system was inhibited (Groups OA and SA), the action of a subcutaneous pump (Alzet, Durect Corporation, Cupertino, CA, USA; 2 mL) that continuously administered atropine (10 mg/kg per day)[33,34] diluted in ethanol and saline was initiated on Day 0. Under general anaesthesia, the pump was introduced under the dorsal skin into the back side of the animal, below the thoracic region.

5.2 | Anaesthesia and surgical preparations

On Day 0, the rats were anaesthetized with an intraperitoneal injection of chloral hydrate (5%, 400 mg/kg; Sigma-Aldrich) to allow spontaneous breathing during measurement of EELV.[35] To measure respiratory mechanics with the FOT (see below), the rats were then intubated (Portex ED 2.4 mm, ID 1.7 mm connected to Abbocath-T 14G) and mechanically ventilated (a tidal volume of 7 mL/kg, a respiratory rate of 70 breaths/minute and a positive end-expiratory pressure (PEEP) of 2.5 cmH_2O; Harvard Apparatus Inc. Holliston, MA, USA; Model 683 Rodent Ventilator). To minimize surgical intervention, the rats were equipped with a superficial iv line in the femoral area with a catheter (Vasculon 26G, BD Allschwil, Switzerland), and anaesthesia was maintained with an iv infusion of midazolam-fentanyl (both from Sintetica SA, Mendrisio, Switzerland) and atracurium (Labatec-Pharma, Geneva, Switzerland). On Day 21, the same anaesthetic protocol was performed with an additional catheterization

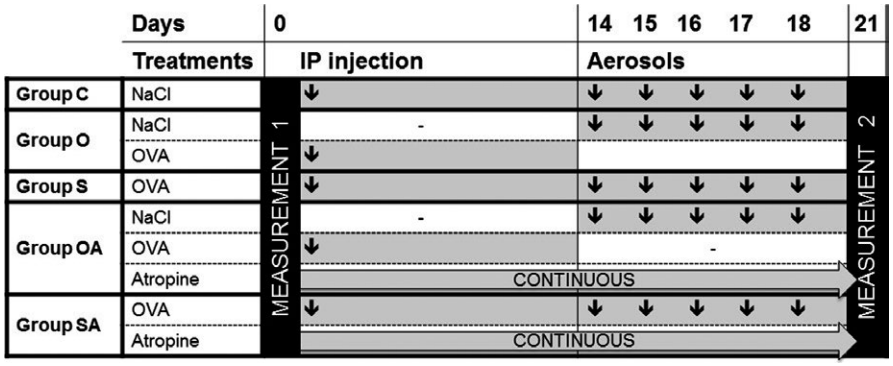

FIGURE 5 Summary of the protocol groups and treatments in the sensitization period. NaCl, normal saline vehicle; OVA, ovalbumin; IP, intraperitoneal. Arrows indicate timing of interventions in the different groups

of the femoral vein for administration of the anaesthetic drugs and cannulation of the femoral artery to register the systemic blood pressure during the experiment (Abbocath-T 22G Abbott Medical, Zurich, Switzerland; acquisition data BIOPAC System MP100 ACE, Goleta, CA, USA). During both anaesthetic procedures, the animals were monitored with an electrocardiograph, allowing adaptation to the depth of anaesthesia. Body temperature was monitored with a rectal thermometer, and a heating pad (Harvard Apparatus Homeothermic Monitor for small animals, Holliston, MA, USA) was applied to maintain the temperature in the normal range (37 ± 0.5°C).

5.3 | Experimental protocol

The experimental protocol was designed to study EELV and MCh (Bichsel, Interlaken, Switzerland) responsiveness in a follow-up manner, while the OVA provocations were limited to the second set of experiments.

The rats were anaesthetized and ventilated on Day 0, and EELV and baseline forced oscillatory respiratory mechanical measurements were performed. MCh provocations were then induced with increasing doses of iv MCh (4, 8 and 16 μg/kg per minute). After a stabilization period allowing a steady-state response to develop (8–13 minutes), the changes in respiratory mechanics were assessed at 1-minute intervals by collecting at least three reproducible forced oscillatory recordings. After the highest dose of MCh, a 15-minute period was allowed for the animals to recover; this ensured that there was no residual effect of MCh. The sensitization and treatment protocol was then performed on each animal.

On Day 21, the same experimental procedure was repeated by re-establishing the baseline EELV and respiratory mechanics, together with a repeated assessment of the MCh responsiveness. Bilateral vagotomy was then performed in the mid-cervical region. A second provocation challenge with the specific allergen OVA was then performed (2 mg iv).[36] Three forced oscillatory recordings were collected at baseline, and individual oscillatory impedance data were then measured at 30-second intervals after the injection of the allergen for 7 minutes or until a systemic hemodynamic collapse (mean systemic blood pressure reaching ~30 mm Hg). Finally, BALF was collected for the assessment of bronchial inflammation.

5.4 | Measurements

5.4.1 | End-expiratory lung volume

To assess EELV, the rats were placed in a supine position in a sealed Plexiglas chamber (2.8 L) and were ventilated at a PEEP of 2.5 cmH_2O, as detailed previously.[37] The mechanical ventilation was suspended before the registrations. At the time of suspension, the plethysmograph was opened to the atmosphere with the trachea opened to a pressurized chamber to equilibrate the lungs to a pressure of 2.5 cmH_2O. The plethysmograph box was then closed and the pressures in the trachea (Ptr) and in the plethysmograph box (Pbox) were recorded simultaneously by miniature pressure transducers (Model 33NA002D; ICSensors, Milpitas, CA, USA) while the rats made spontaneous breathing efforts. Recordings included 6–8 breathing manoeuvres in a 10-second measurement period. Corrections for the thermal properties of the plethysmograph were made in the recordings of Pbox. The corresponding changes in Ptr and Pbox were used to calculate EELV on the basis of Boyle's law.[37] For an exact comparison, the EELV data obtained on Days 0 and 21 were normalized to the bodyweights of the animals.

5.4.2 | Respiratory mechanics

Forced oscillation technique was applied to assess the contributions of the airway and tissue mechanical properties to the total respiratory resistance, as detailed previously.[38,39] Briefly, the mechanical impedance of the respiratory system (Zrs) was measured during short apnoeic periods while the tracheal cannula was connected from the respirator to a loudspeaker-in-box system at end-expiration. A pseudorandom forced oscillatory pressure signal was delivered by the loudspeaker (frequency range 0.5–21 Hz) via a wave-tube (2 mm internal diameter, length 102 cm). Two identical pressure transducers (Model 33NA002D; ICSensors, Milpitas, CA, USA) were used to measure the lateral pressures at the loudspeaker and at the tracheal end of the wave-tube, and Zrs was calculated from a fast Fourier transformation of the pressure-transfer function as the load impedance of the wave-tube.[38]

To assess the changes in the airway and tissue mechanical properties separately, a mathematical model containing a frequency-independent airway resistance (Raw) and inertance (Iaw) connected to a constant-phase tissue compartment,[40] including damping (G) and elastance (H), was fitted to the Zrs spectra by minimizing the differences between the measured and modelled impedance values:

$$Zrs = Raw + j\omega Iaw + (G - jH)/\omega^{\alpha},$$

where j is the imaginary unit, ω is the angular frequency (2πf), and α = 2/π arctan(H/G). It has been established that the Raw parameter is primarily related to the overall airway geometry, as the contribution of the chest wall to the frequency-independent Newtonian resistance is minor.[41] The parameters G and H reflect the energy losses (damping or resistance) and the energy storage (stiffness or elastance) of the total respiratory system, respectively.[41]

5.4.3 | Bronchoalveolar lavage fluid

To evaluate the inflammatory lung response, we analysed the BALF. At the end of the second experiment, the trachea of the rat was connected to a tube (Original Perfusor-Leitung PE, Braun 50 cm, Discofix 35C) attached to a container (Syringe Omnifix 10 mL; Sigma-Aldrich, Buchs, Switzerland) filled with phosphate-buffered saline (PBS) positioned at a height of 20 cm, and filling was subsequently performed. When the hydrostatic filling reached a volume of 10 mL, the BALF was retained. The total number of cells in the BALF was counted by using a Neubauer chamber.

The BALF was centrifuged in a 2 mL Eppendorf tube (750 rpm for 7 minutes). The supernatant was removed, and the pellet was re-suspended in an adequate quantity of PBS[42] to obtain a concentration of 10^4 cells/100 μL. For cytological evaluation, a cytospin was performed with 100 μL of the suspended cells. The slides were fixed and stained with May-Grünwald-Giemsa solution (Sigma-Aldrich). Slices were scanned with a Mirax system (3DHistech, Budapest, Hungary). A collaborator who was uninformed of the experimental protocol manually counted a selected area inside the spot (with a Panoramic Viewer; 3DHistech) and discriminated the following cell types: eosinophil, macrophage, polymorphonuclear and lymphocyte. The total number of cells in the spot area was obtained by multiplying these counts by the total surface area.

5.5 | Statistical analyses

Data are presented as means ± SEM. The Shapiro-Wilk test was used to test data for normality, data were normalized by using a logarithmic transformation if normality test failed. Two-way repeated measures ANOVA tests, with time (Day 0 and Day 21) as the within group and the group allocation (C, O, S, OA and SA) as the independent factor, were performed to evaluate the differences in bodyweight and EELV between the protocol groups and to assess the effects of OVA on the respiratory mechanical parameters. Two-way repeated measures ANOVA tests, with within group variables of MCh dose (baseline, 4, 8 and 16 μg/kg per minute) and between group factor of protocol groups (C, O, S, OA and SA), were used to evaluate the effects of treatments on lung responsiveness to the bronchoconstrictor provocation. Holm-Sidak tests were used for post hoc analyses. Data obtained from the BALF were not normally distributed, these data were analysed by using one-way ANOVA on ranks. $P < 0.05$ was considered statistically significant.

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CONFLICT OF INTERESTS

The authors have no related conflict of interests to declare.

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